Exhibit 11

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Multiple WW Domains, but Not the C2 Domain, Are Required for Inhibition of the Epithelial Na^+ Channel by Human Nedd4*

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The epithelial Na^+ channel (ENaC) absorbs Na^+ across the apical membrane of epithelia. The activity of ENaC is controlled by its interaction with Nedd4; mutations that disrupt this interaction increase Na^+ absorption, causing an inherited form of hypertension (Liddle's syndrome). Nedd4 contains an N-terminal C2 domain, a C-terminal ubiquitin ligase domain, and multiple WW domains. The C2 domain is thought to be involved in the Ca^{2+}-dependent localization of Nedd4 at the cell surface. However, we found that the C2 domain was not required for human Nedd4 (hNedd4) to inhibit ENaC in both *Xenopus* oocytes and Fischer rat thyroid epithelia. Rather, hNedd4 lacking the C2 domain inhibited ENaC more potently than wild-type hNedd4. Earlier work indicated that the WW domains bind to PY motifs in the C terminus of ENaC. However, it is not known which WW domains mediate this interaction. Glutathione *S*-transferase-fusion proteins of WW domains 2–4 each bound to α, β, and γENaC *in vitro*. The interactions were abolished by mutation of two residues. WW domain 3 (but not the other WW domains) was both necessary and sufficient for the binding of hNedd4 to αENaC. WW domain 3 was also required for the inhibition of ENaC by hNedd4; inhibition was nearly abolished when WW domain 3 was mutated. However, the interaction between ENaC and WW domain 3 alone was not sufficient for inhibition. Moreover, inhibition was decreased by mutation of WW domain 2 or WW domain 4. Thus, WW domains 2–4 each participate in the functional interaction between hNedd4 and ENaC in intact cells.

The epithelial Na^+ channel (ENaC)[1] forms the pathway for Na^+ absorption across epithelia, where it plays a critical role in Na^+ homeostasis (1, 2). Nedd4 decreases Na^+ current by enhancing the rate of ENaC degradation; as a result, there are fewer Na^+ channels at the cell surface (3, 4). This regulation is dependent on the ubiquitin ligase activity of Nedd4, suggesting that Nedd4 might inhibit ENaC via channel ubiquitination (3). Consistent with this hypothesis, mutation of potential ubiquitination sites in ENaC increased Na^+ current (5).

Nedd4 contains multiple WW domains, three in rat and mouse (6, 7) and four in human and *Xenopus* (4, 8). Previous studies found that the WW domains bind to PY motifs in the C termini of α, β, and γENaC (6, 8, 9), mediating a direct physical interaction between these proteins. ENaC mutations that disrupt this interaction increase renal Na^+ absorption (3, 6, 10, 11), causing an inherited form of hypertension (Liddle's syndrome) (12). Thus, the interaction between Nedd4 and ENaC is critical for Na^+ homeostasis and blood pressure regulation. An important unresolved question is which WW domain or domains mediate the interaction with ENaC. *In vitro*, multiple WW domains have the capacity to interact with ENaC PY motifs. For example, all three rat WW domains interacted with ENaC using an *in vitro* binding assay (6). In contrast, mouse and human WW domain 1 did not interact with ENaC (8, 9). However, it is not known which WW domains interact with ENaC in the intact cell.

At the N terminus, Nedd4 has a C2 domain, first described as a regulatory domain in protein kinase C (13). Homologous sequences were subsequently identified in a number of other proteins, where they function in the binding and modulation of protein function by Ca^{2+} and phospholipids (14). In Nedd4, the function of the C2 domain is unknown. Previous work found that increased cytosolic Ca^{2+} induced the translocation of a C2 domain-glutathione *S*-transferase (GST) fusion protein to the cell surface, possibly by interacting with annexin XIIIb (15, 16). This suggests that the C2 domain might function in the Ca^{2+}-dependent localization of Nedd4. Consistent with such a model, ENaC is inhibited by increases in cytosolic Ca^{2+} (2). However, the role of the C2 domain in the Nedd4-mediated inhibition of ENaC is not known.

The goal of this work was to identify the sequences in human Nedd4 (hNedd4) that are necessary for the inhibition of ENaC. First, we tested the requirement for the C2 domain using a hNedd4 construct lacking the C2 domain. Second, we asked which of the four hNedd4 WW domains participate in the inhibition of ENaC. We used two different expression systems with distinct advantages: (*a*) *Xenopus* oocytes, and (*b*) Fischer rat thyroid (FRT) epithelia. Oocytes reconstitute some aspects of the Nedd4-dependent regulation of ENaC (3, 4). Conversely, FRT cells allowed us to study hNedd4-mediated regulation in a polarized epithelium.

EXPERIMENTAL PROCEDURES

DNA Constructs—hNedd4 was cloned by polymerase chain reaction of cDNA reverse transcribed from kidney polyadenylated RNA (CLONTECH). The 5′ primer was CCATCGATGGAGCCATGGCAACTTGCGCGGTGGAGGTG and included a *Cla*I site for cloning into pMT3. The 3′ primer was CAGGGCTTTGATGGAGTTGATTAGGGGGTACCCC and included a *Kpn*I site. A hNedd4 construct lacking the C2 domain (ΔC2) was generated by site-directed mutagenesis (QuickChange; Stratagene)

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[1] The abbreviations used are: ENaC, epithelial Na^+ channel; GST, glutathione *S*-transferase; hNedd4, human Nedd4; I_{SC}, short-circuit current; SEAP, secreted alkaline phosphatase; FRT, Fischer rat thyroid; GFP, green fluorescent protein; TBS, 150 mM NaCl and 50 mM Tris, pH 7.4; PAGE, polyacrylamide gel electrophoresis.

(amino acids 22–114 were deleted). To abolish binding to ENaC, each of the four WW domains was mutated (individually or in combination) at two positions (WW1, V210W and H212G; WW2, V367W and H369G; WW3, I440W and H442G; WW4, I492W and H494G). These mutations were generated in ΔC2 because this construct inhibited ENaC more potently than wild-type hNedd4. Human α, β, and γENaC in pMT3 or pcDNA3 were cloned as described previously (17, 18). A FLAG epitope (DYKDDDDK) was introduced in the extracellular domain (residue 511) of αhENaC (αENaC-FLAG), as described previously (10). This epitope did not alter the function of ENaC. αENaC-FLAG was used for the hNedd4 binding assay, as described below. Each cDNA was sequenced in the University of Iowa DNA Core Facility.

Expression and Electrophysiology in Xenopus Oocytes—α, β, and γENaC (0.2 ng each) were coexpressed with either wild-type or mutant hNedd4 or with an irrelevant protein (secreted alkaline phosphatase (SEAP); 0.2–0.8 ng) in *Xenopus* oocytes by nuclear injection of cDNA. Expression of SEAP with ENaC did not alter Na^+ current compared with expression of ENaC alone. One day after injection, whole-cell current was measured by two-electrode voltage clamp at −60 mV with oocytes bathed in 116 mM NaCl, 2 mM KCl, 0.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.4). Statistical significance was assessed using Student's unpaired *t* test.

Expression and Electrophysiology in FRT Epithelia—FRT cells were grown on permeable filter supports (Millicell PCF; 0.4 μm, pore size; 12 mm, diameter) in F-12 Coon's media (Harlan) with 5% fetal calf serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, as described previously (19). One day after seeding, cells were cotransfected with α, β, and γENaC (0.07 μg each) and wild-type or mutant hNedd4 or green fluorescent protein (GFP) as a negative control (0.8 μg). The total DNA was held constant by varying the ratio of hNedd4 and GFP. Expression of GFP did not alter ENaC Na^+ currents. The plasmids were mixed with TFX 50 (Promega; 7.9 μg/millicell) in 360 μl/millicell serum-free F-12 Coon's media for 15 min and transferred to the apical surface of the monolayer. One h later, the apical media were replaced with F-12 Coon's media containing 5% fetal calf serum and amiloride (10 μM).

Na^+ transport was measured 2–3 days after transfection in modified Ussing chambers (Jim's Instruments, Iowa City, IA). The apical and basolateral surfaces were bathed in 135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 10 mM dextrose, and 10 mM HEPES (pH 7.4) at 37 °C and bubbled with O_2. Amiloride-sensitive short-circuit current (I_{SC}) was determined as the difference in current with and without amiloride (10 μM) in the apical bathing solution.

Immunoprecipitation of hNedd4—cDNA encoding wild-type or mutant hNedd4 (0.2 ng) was expressed in *Xenopus* oocytes as described above. Oocytes not injected with cDNA were used as a negative control. One day after injection, 15 oocytes were incubated in modified Barth's solution containing 1 mCi/ml [^{35}S]methionine/cysteine for 4 h and then homogenized in 800 μl of TBS containing 1% Triton X-100 and protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotonin, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A) by 10 pulls through an 18-gauge needle, followed by 1 pull through a 26-gauge needle. Yolk pellets were removed by centrifugation at 5,000 rpm for 5 min and then at 14,000 rpm for 5 min. hNedd4 was immunoprecipitated from the lysate with polyclonal sheep antisera (1:100 dilution) prepared against GST-WW domain fusion proteins (GST-WW1, GST-WW2, or GST-WW3 described below) (Elmira Biologicals, Iowa City, IA). After separation by SDS-PAGE, the proteins were imaged by fluorography and quantitated using a Kodak Image Station 440CF and 1D software (version 3.0, Kodak).

GST-WW Domain Fusion Protein Binding Assay—cDNAs encoding WW domains 1–4 (wild-type or mutant as described above) were generated by polymerase chain reaction and ligated into pGEX-2TK (Amersham Pharmacia Biotech). The sequences correspond to the WW domains shown in Fig. 2*B*. GST-WW domain proteins were expressed and isolated with glutathione-Sepharose beads. Each produced a single band of the expected molecular weight on a Coomassie Blue-stained gel. Protein was quantitated with a Bio-Rad protein assay, and 50 μg of protein was electrophoresed by SDS-PAGE and transferred to a nitrocellulose membrane, and the membrane was blocked overnight with 5% dry milk in TBS containing 0.1% Triton X-100 (Pierce). [^{35}S]Methionine-labeled α, β, and γENaC were transcribed and translated *in vitro* (Promega TNT kit). The membranes were incubated with 25 μl of one of the three ENaC subunits in 25 ml of TBS with 0.1% Triton X-100, 5% bovine serum albumin, and 0.5% dry milk overnight at 4 °C (with rocking), washed four times with TBS/0.1% Triton X-100, and exposed to Kodak Biomax film. To examine the relative binding, GST-WW fusion proteins (2 μg) were applied directly to nitrocellulose using the Bio-Dot SF apparatus (Bio-Rad) and probed with α, β, or γENaC as described above.

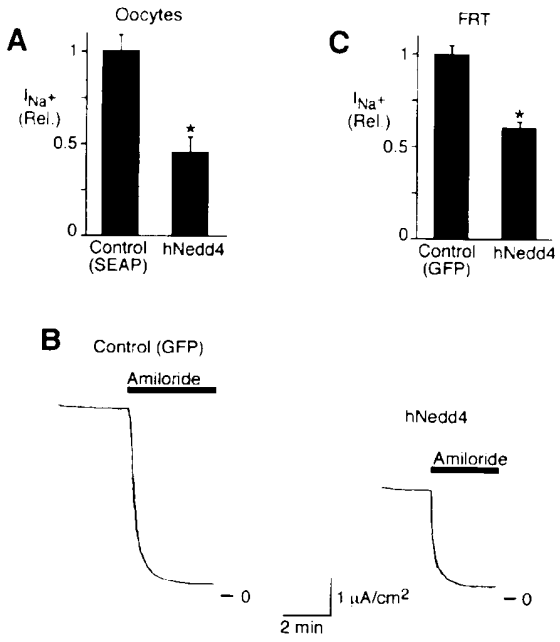

FIG. 1. **Inhibition of ENaC by hNedd4.** *A*, amiloride-sensitive whole-cell Na^+ current (relative to control) at −60 mV in *Xenopus* oocytes coexpressing α, β, and γENaC (0.2 ng each) with hNedd4 or SEAP (0.8 ng) (mean ± S.E.; $n = 15$). *, $p < 0.003$. *B*, representative time course of I_{SC} in FRT epithelia expressing α, β, and γENaC with hNedd4 or GFP (negative control). Amiloride (10 μM) was added to the apical membrane, as indicated by the *bars*. *C*, amiloride-sensitive I_{SC} (relative to control) in FRT epithelia expressing α, β, and γENaC (0.07 μg each) with hNedd4 or GFP (0.8 μg) (mean ± S.E.; $n = 17$). *, $p < 0.0001$.

hNedd4 Binding Assay—αENaC-FLAG was expressed in COS-7 cells by electroporation, as described previously (10). Two days after electroporation, the cells were lysed, and protein was solubilized in TBS containing 1% Triton X-100 and protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotonin, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A). Insoluble protein was solubilized in 2% SDS, 1 mM EDTA, 1% 2-mercaptoethanol, and 10 mM Tris (pH 7.4) and diluted 1:10 (1 ml, final volume) in TBS/1% Triton X-100. αENaC-FLAG was immunoprecipitated from 100 μl of lysate (0.74 μg/μl total protein) with anti-FLAG M2 monoclonal antibody (1:1,000; Kodak) and protein A beads (Pierce). The immunoprecipitated αENaC-FLAG could be detected either by metabolic labeling of the cells or by immunoblotting with anti-FLAG M2 antibody (data not shown).

hNedd4 (wild-type or mutant) was generated and labeled with [^{35}S]methionine by *in vitro* transcription and translation. After SDS-PAGE and fluorography, the protein was quantitated using Kodak Image Station and 1D software. The immunoprecipitated αENaC-FLAG (30 μl immobilized on beads) was then incubated for 16 h with wild-type or mutant hNedd4 (equal specific activities, 12.8–20 μl) in a total volume of 800 μl. The beads were washed three times with TBS/1% Triton X-100, separated by SDS-PAGE, and imaged by fluorography.

RESULTS

Inhibition of ENaC by hNedd4—We tested the effect of hNedd4 on ENaC in two different expression systems. In *Xenopus* oocytes, expression of α, β, and γENaC generated amiloride-sensitive Na^+ current. Coexpression of the channel with hNedd4 decreased Na^+ current (Fig. 1*A* and Ref. 8). To determine whether hNedd4 inhibits ENaC in epithelia, we transiently expressed the channel with or without hNedd4 in FRT epithelial cells. These cells lack endogenous Na^+ channels and form a polarized epithelium when grown on permeable filter supports (19, 20). Transfection of FRT epithelia with α, β, and γENaC using cationic lipids generated transepithelial short-

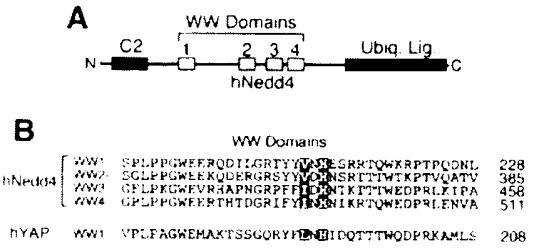

FIG. 2. **hNedd4.** *A*, schematic representation showing the location of the C2 domain, the four WW domains, and the ubiquitin ligase domain. *B*, line-up of amino acid sequences of the four hNedd4 WW domains and human Yes-associated protein. *Right*, the number of the last amino acid in each WW domain is shown. The residues mutated to abolish binding to ENaC are indicated by *black boxes*.

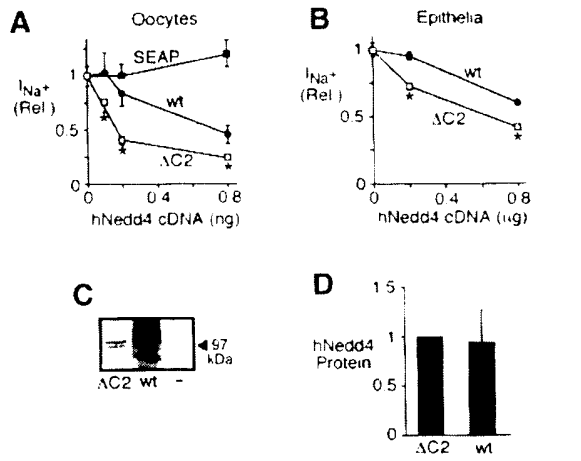

FIG. 3. **Deletion of the C2 domain.** *A*, relative amiloride-sensitive whole-cell Na^+ current at −60 mV in *Xenopus* oocytes coexpressing α, β, and γENaC (0.2 ng each) with wild-type hNedd4 (*wt*), hNedd4 lacking the C2 domain (ΔC2), or SEAP as a negative control (0–0.8 ng) (mean ± S.E.; $n = 6$–14).*, $p < 0.05$ *versus* wild-type hNedd4. *B*, relative amiloride-sensitive I_{Sc} in FRT epithelia expressing α, β, and γENaC (0.07 μg each) with wild-type or ΔC2 hNedd4 (0–0.8 μg) (mean ± S.E.; $n = 12$). In some cases, error bars are hidden by data symbols. *, $p < 0.001$ *versus* wild-type hNedd4. *C*, immunoprecipitation of ΔC2 or wild-type hNedd4 (or uninjected cells as a negative control (−)) with a polyclonal antibody to WW domain 2 in *Xenopus* oocytes labeled with [^{35}S]methionine/cysteine. Preimmune serum did not immunoprecipitate hNedd4 (data not shown). In both lanes, faster-migrating bands were also present, either as a result of degradation or initiation of translation at downstream methionines. The intensity of these bands was variable and always less than that of the band corresponding to the full-length protein (see Fig. 6*C* for examples). *D*, quantitation of hNedd4 protein (relative to ΔC2) using Kodak Image Station and 1D software (mean ± S.E.; $n = 3$). The upper band corresponding to full-length protein was quantitated.

circuit Na^+ current (I_{Sc}) that was completely blocked by amiloride (19) (Fig. 1*B*, *Control*). hNedd4 decreased Na^+ current when coexpressed with ENaC (Fig. 1, *B* and *C*, *hNedd4*). Thus, similar to *Xenopus* oocytes, hNedd4 inhibited ENaC in epithelial cells.

Requirement for the hNedd4 C2 Domain—The N terminus of hNedd4 contains a C2 domain (Fig. 2*A*). To test whether the C2 domain is required for the hNedd4-mediated inhibition of ENaC, we generated a hNedd4 lacking the C2 domain (ΔC2). If this domain was required, we predicted that ΔC2 would not inhibit ENaC. We coexpressed ENaC in *Xenopus* oocytes with increasing amounts of wild-type hNedd4, ΔC2, or an irrelevant protein (SEAP). Wild-type hNedd4, but not SEAP, produced a dose-dependent decrease in Na^+ current (Fig. 3*A*). Surprisingly, deletion of the C2 domain did not prevent hNedd4 from inhibiting ENaC (Fig. 3*A*). On the contrary, ΔC2 was a more potent inhibitor of ENaC than wild-type hNedd4. This difference did not result from altered protein expression; immunoprecipitation with a polyclonal antibody against WW domain 2 revealed similar amounts of protein for wild-type and mutant hNedd4 (Fig. 3, *C* and *D*). We also tested the effect of ΔC2 in FRT epithelia. Similar to the results seen in oocytes, ΔC2 inhibited ENaC more potently than wild-type hNedd4 (Fig. 3*B*). Together, these results indicate that the C2 domain is not required for the hNedd4-mediated inhibition of ENaC. Rather, the data suggest that the C2 domain may suppress the activity of hNedd4.

Interaction between hNedd4 GST-WW Domain Fusion Proteins and ENaC—hNedd4 contains four WW domains (Fig. 2, *A* and *B*). To investigate the interactions between hNedd4 and ENaC, we used an overlay assay. GST fusion proteins containing individual WW domains (GST-WW1, GST-WW2, GST-WW3, and GST-WW4; 50 μg) were electrophoresed, transferred to nitrocellulose membranes (Fig. 4*A*, *bottom panel* shows Coomassie Blue staining), and probed with [^{35}S]methionine-labeled α, β, or γENaC. Consistent with previous work (8), we found that WW domains 2, 3, and 4 each interacted with α, β, and γENaC (Fig. 4*A*). In contrast, WW domain 1 and GST alone (negative control) did not interact with the ENaC subunits (Fig. 4*A*). In Fig. 4*B*, smaller quantities of fusion proteins (2 μg) were applied to nitrocellulose membranes and probed with ENaC. GST-WW3 bound more of the α, β, and γENaC probes than did GST-WW2 or GST-WW4.

Espanel and Sudol (21) recently identified two WW domain residues (Leu-190 and His-192) involved in the binding of Yes-associated protein to a PY motif peptide. Mutation of both residues abolished the interaction. The residue corresponding to His-192 in Yes-associated protein is conserved in each of the hNedd4 WW domains (Fig. 2*B*). At the position equivalent to Leu-190, the hNedd4 WW domains contain conservative substitutions, valine (WW1 and WW2) or isoleucine (WW3 and WW4). To determine whether these residues are required for the interaction between the hNedd4 WW domains and ENaC PY motifs, we generated GST-WW fusion proteins containing mutations in both residues (Val/Ile to Trp; His to Gly). Using an overlay assay, we found that these mutations abolished binding of WW domains 2, 3, and 4 to each ENaC subunit (α, β, and γ) (Fig. 4*A*).

Interaction between Full-length hNedd4 and ENaC—We took advantage of these mutations to determine the role of each WW domain in the binding of full-length hNedd4 to ENaC. We mutated one or more WW domains and tested the binding of hNedd4 to one of the ENaC subunits (αENaC). αENaC-FLAG was expressed in COS-7 cells, immunoprecipitated, and then incubated with wild-type or mutant hNedd4 (labeled with [^{35}S]methionine). Fig. 5 shows autoradiograms of the *in vitro* translated hNedd4 probes (*bottom panel*) and hNedd4 that bound to αENaC-FLAG (*top panel*). We found that wild-type hNedd4 bound to αENaC-FLAG, but not to immunoprecipitated proteins from COS-7 cells expressing GFP as a negative control (*wt-GFP*). Deletion of the C2 domain (ΔC2) did not disrupt binding. The WW domain mutations were therefore generated in a construct lacking the C2 domain. Simultaneous mutation of all four WW domains (WW1–4) abolished the binding of hNedd4 to αENaC-FLAG, confirming a role for one or more of the WW domains in the interaction between these proteins. Mutation of WW domain 3 alone was sufficient to abolish binding, but individual mutations of WW domains 1, 2, or 4 were not sufficient to abolish binding. However, these mutations appeared to decrease the binding of hNedd4 to αENaC-FLAG (compared with ΔC2). Thus, WW domain 3, but not WW domains 1, 2, or 4, is required for the interaction between hNedd4 and αENaC.

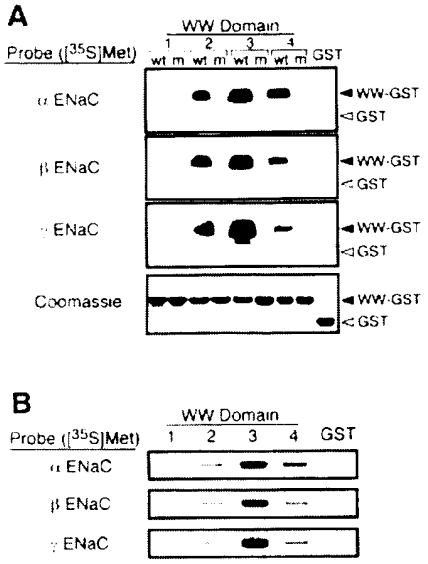

FIG. 4. **Binding of ENaC to GST-WW domain fusion proteins.** *A*, wild-type (*wt*) or mutant (*m*; Val/Ile to Trp; His to Gly) GST-WW domain fusion proteins (or GST alone, 50 μg) were electrophoresed by SDS-PAGE, transferred to nitrocellulose, probed with *in vitro* translated ([^{35}S]methionine-labeled) α, β, or γENaC, and imaged by fluorography. The *bottom panel* shows wild-type and mutant GST-WW domain fusion proteins and GST alone on a Coomassie Blue-stained gel. *B*, 2 μg of the GST-WW domain fusion proteins was bound to nitrocellulose and probed with α, β, or γENaC as described above. Results are representative of four experiments.

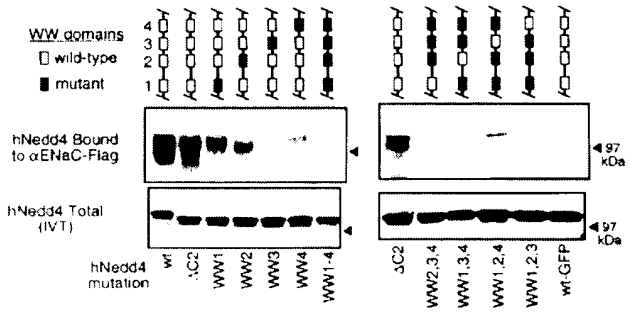

FIG. 5. **Binding of hNedd4 to αENaC.** Autoradiograms of hNedd4 (wild-type and mutants) bound to immunoprecipitated αENaC-FLAG (*top panels*) and total hNedd4 probes (5 μl; *bottom panels*). The hNedd4 constructs contained mutations (Val/Ile to Trp; His to Gly) in the indicated WW domains (schematic representations are shown). These constructs also lacked the C2 domain. αENaC-FLAG was expressed in COS-7 cells (or GFP as a negative control, *wt-GFP*), immunoprecipitated with anti-FLAG M2 antibody, and incubated with wild-type or mutant hNedd4 (equal specific activities of each). The hNedd4 proteins were generated and labeled with [^{35}S]methionine by *in vitro* transcription and translation. hNedd4 that bound to αENaC-FLAG was separated by SDS-PAGE and imaged by fluorography. Autoradiograms are representative of three experiments.

To determine whether individual WW domains were sufficient to interact with αENaC-FLAG, we simultaneously mutated three of the four WW domains (leaving one WW domain intact) (Fig. 5, *right*). When WW domains 1, 2, or 4 alone were intact, we did not detect significant binding between hNedd4 and αENaC-FLAG. Conversely, hNedd4 bound to αENaC-FLAG when WW domain 3 alone was intact (WW domains 1, 2, and 4 were mutated). Together, the data indicate that WW domain 3 is necessary and sufficient for hNedd4 to interact with the αENaC subunit. This is consistent with our finding that more GST-WW3 than the other WW domain fusion proteins bound to each ENaC subunit (Fig. 4). Although WW domains 1, 2, and 4 were not sufficient alone or in combination for full-length hNedd4 to bind αENaC-FLAG, the data to not exclude a role for these WW domains in the interaction of hNedd4 with the ENaC channel complex. We therefore used two functional assays to determine the role of the four WW domains in the interaction between hNedd4 and ENaC in the intact cell.

WW Domain Requirements for the Inhibition of ENaC—To determine which WW domains are required for hNedd4 to inhibit ENaC in the intact cell, we coexpressed α, β, and γENaC with hNedd4 cDNAs containing WW domain mutations (described in the previous section). These mutations were generated in ΔC2 hNedd4 because it inhibited ENaC better than did wild-type hNedd4. We found that simultaneous mutation of all four WW domains (WW1–4) abolished hNedd4-mediated inhibition of ENaC in FRT epithelia (Fig. 6, *A* and *B*). Similar results were obtained in *Xenopus* oocytes (Fig. 6*B*). Thus, the interaction between one or more of the WW domains and ENaC was required for inhibition. To identify which of the four WW domains was required, we tested individual WW domain mutations. Mutation of WW domain 1 did not prevent hNedd4 from inhibiting ENaC in either FRT epithelia or *Xenopus* oocytes (Fig. 6*B*). This is consistent with the finding that WW1 did not bind to ENaC *in vitro* (Figs. 4 and 5). Interestingly, the WW1 mutant inhibited ENaC slightly (but significantly) better than did hNedd4 with wild-type WW domains (Fig. 6*B*). In contrast, mutation of WW3 nearly abolished inhibition; expression of the WW3 mutant in epithelia or oocytes produced a minimal decrease in Na^+ current (Fig. 6*B*). Mutation of WW2 or WW4 had an intermediate effect. In epithelia, both mutants inhibited ENaC, but they did so to a lesser extent than did ΔC2 containing wild-type WW domains (Fig. 6*B*). Although mutation of WW2 also decreased inhibition in *Xenopus* oocytes, the WW4 mutant inhibited ENaC to an extent similar to that of ΔC2 (Fig. 6*B*). The WW domain mutations did not alter the expression of hNedd4 proteins in *Xenopus* oocytes (Fig. 6, *C* and *D*). Thus, WW3 is required for hNedd4-mediated inhibition of ENaC. WW2 and WW4 are not required, but they are also involved in ENaC inhibition. The data are consistent with our observation that WW3 was required for the interaction between hNedd4 and ENaC (Fig. 5) and also suggest a functional role for WW domains 2 and 4.

Our binding studies suggested that WW domain 3 was sufficient alone to bind to ENaC (Figs. 4 and 5). To test whether this interaction would inhibit ENaC, we expressed the channel with hNedd4 containing mutations in WW domains 1, 2, and 4 (WW3 alone was intact). Surprisingly, the mutant hNedd4 decreased Na^+ current only 20% in FRT epithelia, much less than did ΔC2 hNedd4 (Fig. 7*A*). This result could not be explained by decreased hNedd4 protein; similar amounts of ΔC2 and mutant hNedd4 protein were expressed (Fig. 7*B*). Current was also minimally inhibited when only WW domain 2 was intact and was not inhibited at all when WW domain 4 alone was intact (Fig. 7*A*). Thus, the interaction between a single WW domain and ENaC produced only minimal inhibition of ENaC.

DISCUSSION

WW domains in hNedd4 interact with PY motifs in ENaC (6, 8, 9). We found that simultaneous mutation of all four WW domains abolished the inhibition of ENaC by hNedd4. In previous work, inhibition was also abolished by mutation or deletion of ENaC PY motifs (3, 4). Thus, a physical interaction between one or more WW domain(s) and the PY motifs of ENaC is required for hNedd4 to inhibit the channel. Although three of the four hNedd4 GST-WW domain fusion proteins interacted with the PY motifs of ENaC *in vitro*, several findings suggest

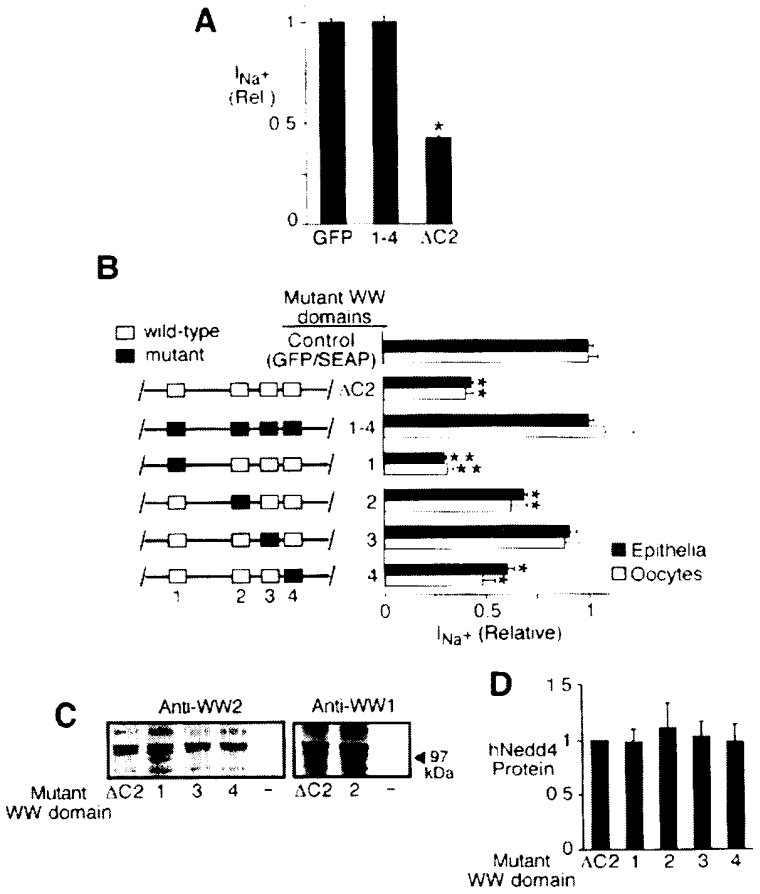

FIG. 6. **Effect of WW domain mutations on hNedd4-mediated inhibition of ENaC.** *A*, amiloride-sensitive I_{Sc} (relative to GFP control) in FRT epithelia expressing α, β, and γENaC (0.07 μg each) with hNedd4 ΔC2 (ΔC2), hNedd4 with mutations in all four WW domains (*1–4*), or GFP (0.8 μg; mean ± S.E.; n = 20). *, $p < 0.0001$ *versus* control (GFP). *B*, relative amiloride-sensitive I_{Sc} in FRT epithelia and amiloride-sensitive whole-cell Na^+ current at −60 mV in *Xenopus* oocytes coexpressing α, β, and γENaC (0.07 μg and 0.2 ng each in FRT and oocytes, respectively) with an irrelevant control protein (GFP in epithelia; SEAP in oocytes), hNedd4 ΔC2, or hNedd4 ΔC2 with mutations in the indicated WW domain(s) (0.8 μg and 0.2 ng in FRT and oocytes, respectively) (mean ± S.E.; n = 16–20). Schematic indicates the wild-type and mutant WW domains. *, $p < 0.0001$ *versus* control; **, $p < 0.0001$ *versus* control and $p < 0.05$ *versus* ΔC2. *C*, immunoprecipitation of the indicated hNedd4 proteins expressed in *Xenopus* oocytes (or uninjected cells (−)) with polyclonal antibodies to WW domain 2 or WW domain 1, as indicated. *D*, quantitation of hNedd4 proteins containing the indicated mutant WW domains (relative to ΔC2) (mean ± S.E.; n = 3–7).

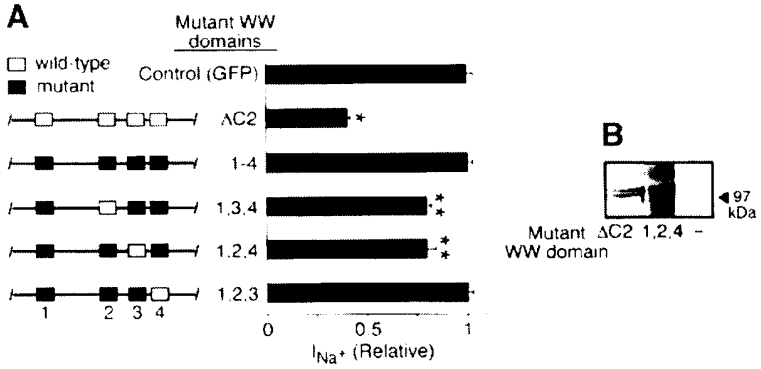

FIG. 7. **Single intact WW domains.** *A*, relative amiloride-sensitive I_{Sc} in FRT epithelia expressing α, β, and γENaC (0.07 μg each) with GFP, hNedd4 ΔC2 or hNedd4 ΔC2 with the indicated mutant WW domains (0.8 μg) (mean ± S.E.; n = 18–19). Schematic indicates the wild-type and mutant WW domains. *, $p < 0.0001$ *versus* control; **, $p < 0.003$ *versus* control and $p < 0.0001$ *versus* ΔC2. *B*, immunoprecipitation of the indicated hNedd4 proteins expressed in *Xenopus* oocytes (or uninjected cells (−)) with a polyclonal antibody to WW domain 3.

that the WW domain 3 interaction is critically important. First, GST-WW3 bound more ENaC probe than did GST-WW2 or GST-WW4. This suggests the possibility that WW domain 3 has a higher relative affinity for ENaC than the other WW domains, although we cannot exclude other potential explanations (*e.g.* differences in conformation or aggregation of the fusion proteins). Second, mutation of WW domain 3, but not of the other WW domains, abolished the binding of hNedd4 to an ENaC subunit. Third, WW domain 3 alone was sufficient to mediate the binding of full-length hNedd4 to αENaC. Because GST-WW3 bound well to all three ENaC subunits, it seems likely that WW domain 3 is also important in the binding of hNedd4 to β and γENaC. Finally, the inhibition of ENaC by hNedd4 in both epithelial cells and *Xenopus* oocytes was nearly abolished by mutation of WW domain 3. Thus, WW domain 3 was critical not only for the binding of hNedd4 to ENaC *in vitro* but also for its binding and inhibition of the heteromultimeric channel in the intact cell.

However, the interaction between WW domain 3 and ENaC alone was not sufficient for hNedd4-mediated inhibition of ENaC; hNedd4 produced only a small decrease in Na^+ current when WW domain 3 was the only intact WW domain. This suggests that WW domain 2 and/or WW domain 4 also participate in the functional interaction with ENaC. Consistent with this hypothesis, mutation of WW domain 2 or WW domain 4 decreased ENaC inhibition (although the decrease was less than for the WW domain 3 mutation). Thus, although the interaction with WW domain 3 appears to be most important, WW domains 2, 3, and 4 each participate in the interaction of hNedd4 with ENaC in intact cells. This is also consistent with the previous finding that multiple WW domains were required for a WW domain-GST fusion protein to disrupt the Na^+

(70 mM)-induced inhibition of Na^+ current in mouse mandibular duct cells (9).

An important unresolved question is which of the ENaC PY motifs interact with WW domains 2–4. Mutation or deletion of the PY motif in βENaC was sufficient to disrupt Nedd4-mediated inhibition of the channel and cause Liddle's syndrome (3, 12). However, all three PY motifs have the capacity to interact with WW domains 2, 3, and 4 *in vitro* (8), and mutations in any of the three PY motifs increase Na^+ current (10). Thus, it is possible that the interactions are relatively promiscuous. For example, WW domain 3 might bind to the αENaC PY motif in one channel, but it might bind to the PY motif of βENaC in another channel. Multiple PY motif-WW domain interactions might be required to increase the affinity of the interaction between ENaC and hNedd4. Alternatively, there could be a high degree of specificity in the interaction between WW domains and PY motifs in the intact cell. For example, each WW domain might bind to a different ENaC subunit. Finally, it is possible that WW domains 2–4 bind to PY motifs in different ENaC channels or even different proteins, cross-linking them into a larger complex. Future studies will be required to differentiate between these models. However, each model is consistent with the finding that a single WW domain interaction is not sufficient to inhibit ENaC.

The function of WW domain 1 is not known. It does not bind to ENaC subunits *in vitro* (8), and mutation of WW1 did not prevent hNedd4 from inhibiting ENaC, suggesting that it does not bind to ENaC in the intact cell. Rather, the WW domain 1 mutant inhibited ENaC to a greater extent than did wild-type hNedd4. Perhaps the binding of WW domain 1 to one or more other proteins modulates the localization or function of hNedd4. Identification of such proteins may provide new candidate genes for the pathogenesis of hypertension.

The interaction between WW domain 3 and ENaC was most important for the inhibition of the channel. Interestingly, this domain is not present in rat or mouse Nedd4 (which contain only three WW domains); based on sequence similarity, the three WW domains of rat and mouse Nedd4 correspond to WW domains 1, 2 and 4 in hNedd4 (8). This suggests that the pattern of interactions between the WW domains and PY motifs and the affinity of the interaction between Nedd4 and ENaC may differ between species. Thus, it may be difficult to extrapolate PY motif-WW domain binding data between different species, and it may therefore be particularly important to study human Nedd4 to understand the role of this protein in human blood pressure control and hypertension.

Previous work suggested that the Nedd4 C2 domain may be involved in the Ca^{2+}-dependent translocation of Nedd4 to the cell surface through a mechanism potentially involving an interaction between the C2 domain and annexin XIIIb (15, 16). This might be important for the binding of Nedd4 to ENaC at the cell surface. Such a mechanism could be involved in the regulation of ENaC by cytosolic Ca^{2+}. Surprisingly, we found that the C2 domain was not required for the hNedd4-mediated inhibition of ENaC. Instead, a mutant hNedd4 lacking the C2 domain inhibited ENaC better than did wild-type hNedd4. Perhaps under basal conditions, the C2 domain blocks the interaction and/or inhibition of ENaC by hNedd4. Increased cytosolic Ca^{2+} might relieve this block, resulting in channel inhibition by hNedd4. Deletion of the C2 domain might allow hNedd4 to inhibit the channel in the absence of an increase in Ca^{2+}. Such a model is consistent with the observation that increased cytosolic Ca^{2+} inhibits Na^+ absorption via ENaC. Alternatively, Nedd4 might interact with ENaC at an intracellular location, rather than at the cell surface.

Inhibition of ENaC by hNedd4 plays a critical role in Na^+ homeostasis and blood pressure control. Mutations in ENaC that abolish the interaction between these proteins cause Liddle's syndrome, a genetic form of hypertension. We found that mutations in WW domains 2, 3, and 4 disrupted the ability of hNedd4 to inhibit ENaC. Mutations in the ubiquitin ligase domain also disrupted inhibition (3). Thus, it seems possible that loss of function mutations in hNedd4 could increase renal Na^+ absorption, identifying hNedd4 as a candidate gene for hypertension. An understanding of the molecular requirements for the binding and inhibition of ENaC by hNedd4 will facilitate the search for sequence variations that alter this regulation and may provide new insights into the basic mechanisms of blood pressure control and the pathogenesis of hypertension.

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